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Tumor–associated antigens identified by mRNA expression profiling as tumor rejection epitopes

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Abstract

Thirteen H-2^b-binding peptides derived from six potentially overexpressed proteins in p53$^{-/-}$ thymoma (SM7) cells were studied for immunogenecity and vaccine-induced prevention of tumor growth in mice inoculated with SM7 tumor cells. Six of the peptides generated specific CTL responses after immunization, but only two of these peptides ($RAD_{23–31}$ and $RAD_{24–31}$) were capable of generating a weak vaccination-induced protection against adoptive tumor growth. SM7 inoculated mice treated with a blocking antibody against the inhibitory T cell signal transducing molecule CTLA4 appeared to delay tumor take, suggesting that SM7 thymoma cells are recognized by the adaptive immune system of the host. However, prophylactic vaccination with $RAD_{23–31}$ and $RAD_{24–31}$ peptides combined with anti-CTLA4 Ab treatment and did not improve tumor resistance. Our data would indicate that vaccination with immunogenic peptides derived from potentially overexpressed tumor proteins, as identified by mRNA expression profiling of p53$^{-/-}$ thymoma cells, at best results in a weak tumor protection thus questioning this way of detection of new tumor rejection epitopes.

Introduction

Identification of tumor associated antigens (TAA) recognized by CD8$^+$ T cells and the corresponding major histocompatibility complex class I (MHC-I) restricted epitopes has led to peptide-based vaccination approaches in experimental animals as well as in clinical settings [1–5]. Since many MHC-I restricted TAA so far identified represent peptides derived from self proteins, it is not surprising that most of these TAA are relatively weak immunogens and that reports demonstrating tumor regression after peptide vaccination in clinical trials are sparse. Occasional marked clinical regressions of melanoma have been observed after peptide vaccination [5–7].

To search for new TAA, we have recently used mRNA profiling to analyze a panel of spontaneously arising thymomas in p53$^{-/-}$ mice and identified a number of upregulated mRNAs [8]. Immunizing with a pool of six peptides representing upregulated RAD50, a part of a DNA regulatory protein complex [9], we obtained partially protection

against the take and growth of inoculated tumor cells overexpressing RAD50 mRNA. This finding suggested to us that tumor rejecting epitopes can be identified by mRNA expression profiling.

In the present work we have focussed on the CTL generating effect after immunization with individual RAD50 derived peptides and with $H2^b$-binding peptides derived from other proteins encoded by differentially upregulated mRNAs [8]. By immunization, half of the peptides, including two of the RAD50-derived peptides, were found to induce significant peptide specific CTL responses. However, none of these peptides were capable of eliciting CTL responses against the thymoma cells from which they were derived. Mice vaccinated with the two immunogenic RAD50 peptides were weakly protected against tumor take, whereas vaccination with a pool of the four immunogenic thymoma associated peptides derived from other, potentially upregulated thymoma proteins, did not influence tumor take. Treatment with a blocking antibody against the cytotoxic T lymphocyte antigen CTLA4 [10] has been shown previously to enhance the effect of tumor rejection in mice vaccinated with irradiated tumor cells [11,12]. However, this treatment did not increase peptide vaccine-induced protection against tumor take, suggesting the tumor associated peptides, characterized in the present study, represent at best very weak tumor rejection epitopes.

Results

Generation of CTL responses

Individual RAD50 derived peptides [8] (see Table 1), with a binding affinity (K_D) for $H\text{-}2^b$ at 12–280 nM [13], were injected subcutaneously in Freunds Incomplete Adjuvant (FIA). Splenocytes were recovered 10 days after immunization and challenged in vitro for 5 days with irradiated syngenic spleen cells pulsed with specific peptide (see Materials and Methods). CTL responses were measured against RMA-.S and SM7 target cells pulsed with specific peptide or mock peptide. Mean data for groups of three immunized mice are shown in Figure 1. Only two of the RAD50 peptides, RAD_{23-31} and RAD_{24-31}, with K_D values of 280 and 70 NM respectively [13], induced a CTL response (Fig. 1A) and only immunization with the RAD_{23-31} peptide induced killing of RAD_{24-31}-pulsed SM7 cells (Fig. 1B), whereas unpulsed SM7 cells were not killed by any of the peptide specific CTLs (Fig. 1C). Experiments (not included) showed that CTLs raised against the RAD_{23-31} peptide killed RAD_{24-31} pulsed RMA-S cells to the same extend as RAD_{23-31} pulsed cells whereas CTLs generated against the RAD_{24-31} peptide killed the RAD_{23-31} pulsed RMA-S with only half the efficiency of RAD_{24-31} peptide pulsed cells. These data suggest that some of the CTLs generated against the RAD_{23-31} peptide separately recognize the C-terminal glutamine in the RAD_{23-31} peptide.

In separate experiments mice were immunized with a mixture of non-immunogenic and immunogenic RAD50 peptides. The data (not included) indicated that CTL development against the two admixed immunogenic RAD50 peptides was not impaired by the admixed non-immunogenic peptides indicating that these peptides do not compete out the MHC-I binding of the immunogenic peptides on the surface of APCs during the process of immunization.

Individual peptides derived from six potentially upregulated SM7 proteins not related to RAD50 (Table 1) [8], with K_D values for $H\text{-}2^b$ binding at 2–9500 nM [13], were used for immunization as described above. Fig. 2A shows that immunization with 4 of 8 peptides derived from these proteins induced significant peptide specific CTL generation. The four immunogen peptides have K_D values of 2–155 [13]. Immunization with peptide $CatB_{47-55}$ also induced killing og $CatB_{47-55}$ pulsed SM7 cells whereas unpulsed thymoma cells were not killed by the peptide specific CTLs (Fig. 2C). The resistance of peptide-pulsed SM7 cells to killing by most peptide specific CTLs does not reflect lack of $H\text{-}2^b$ expression, as documented by FACS analysis (data not included).

Vaccination and anti-CTLA4 antibody-induced protection against tumor take

Groups of 7–8 mice were vaccinationated three times with a pool of the six RAD50 peptides or a mixture of the two immunogenic RAD_{23-31} and RAD_{24-31} peptides. The RAD50 peptides were mixed with equal amounts of FIA and a helper peptide, TPPAYRPPNAPIL [14] was included. Control mice received FIA and helper peptide only. Fig. 3 shows the pooled survival curves for two separate experiments. A significant protection against tumor take was obtained in mice vaccinated with a mixture of RAD_{23-31} and RAD_{24-31} peptides ($p < 0.03$). In disagreement with our previous study [8], vaccination with a mixture of the six RAD50 peptides did not offer any protection in these experiments (data not shown). Two of five vaccine protected mice in Fig. 3 were rechallenge with 10^6 tumor cells 3 months after the primary tumor challenge. Two of the mice developed progressing tumors, suggesting low immunological memory for tumor rejection antigens (data not included). Fig. 4 shows data from one of two experiments with mice immunized with a mixture of the 4 immunogenic peptides depicted in Fig. 2. These peptides are derived from four potentially upregulated SM-7 proteins not related to the RAD50 protein (Table 1). No evidence of protection was obtained after immunization with this serie of peptides in the two separate, but identical vaccination series.

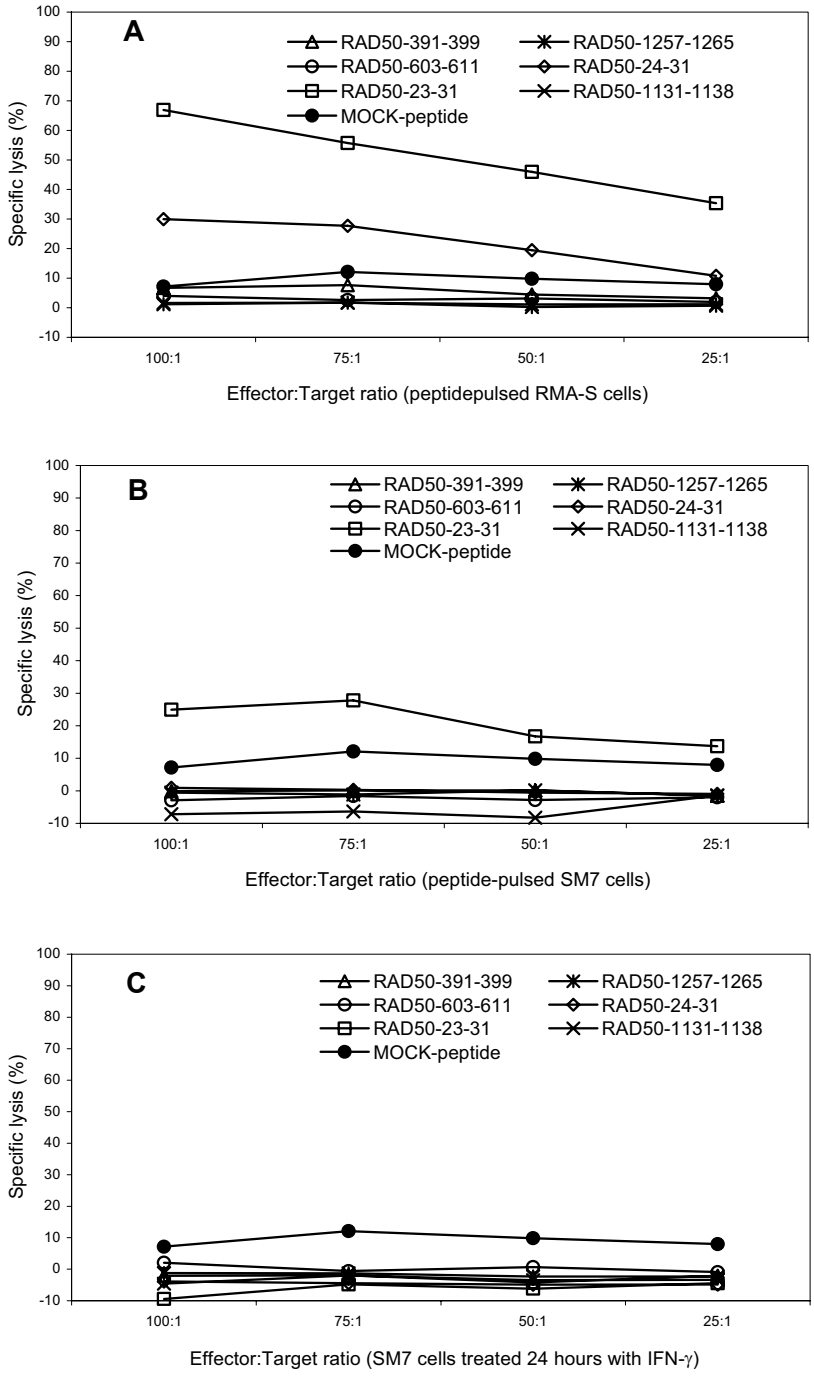

Figure 1
CTL development in vivo against SM7-derived RAD50 peptides. Groups of three mice were immunized once subcutaneously with individual peptides including a helper peptide. Splenocytes were peptide challenged ex vivo at day 10 and assayed for CTL activity five days later. A, Generation of CTLs against RAD50 peptides. B, CTL reactivity against SM7 thymoma cells pulsed with the RAD50 peptides. C, CTL reactivity against non-pulsed SM7 thyoma cells (see Table 1 for peptide name, sequence and MHC-I binding affinity).

Table 1: Potentially overexpressed p53$^{-/-}$ SM7 thymoma proteins as analyzed by mRNA expression profiling including predicted, sequenced and assayed protein-derived H2^{b} binding peptides[a].

Protein name	Peptide sequence	Peptide name	KD nM[b]
RAD50	RQIKNFHEL	RAD50 $_{391–399}$	45
RAD50	SQQRNFQLL	RAD50 $_{603–611}$	12
RAD50	SAEQNKNHI	RAD50 $_{1257–1265}$	13
RAD50	IISFFSPL	RAD50 $_{24–31}$	70
RAD50	QIISFFSPL	RA50 $_{23–31}$	280
RAD50	AIMKFHSM	RAD50 $_{1131–1139}$	36
Endonuclease*	YAYTFWTYM	Encl$_{261–269}$	80
RAD23	KALGFPESL	RAD23 $_{328–336}$	2950
PMS2	LGQFNLGFI	PMS2 $_{676–684}$	410
PMS2*	FGPQDIDEL	PMS2 $_{775–783}$	2
Cathepsin B*	FYNVDIDYL	CatB 47–55	155
Translin	VSEIFVEL	Translin $_{3–10}$	560
Protease-nexin 1*	WHEPFFIL	Pn1 $_{3–10}$	ND
Protease-nexin 1	VHSQFNSL	Pn1 $_{18–25}$	9500

[a]for details see reference no. [8][b]data are from ref. [13]. *Immunogenic peptides, see Figs 1,2.

The data in Fig. 3 and Fig. 4 illustrate that vaccination with the chosen tumor-derived immunogenic peptides results in a very marginal protection against tumor take. In order to investigate whether the growth of SM7 thymoma cells in naïve is controlled by the adaptive immune system, mice challenged with 1 mio. SM7 cells were treated with a CTLA4-blocking antibody. The data in Fig. 5 indicate that this treatment influenced tumor take. Thus antibody treated mice tended to delay tumor take after challenge with 10^6 SM7 cells compared with untreated control mice, the two curves being statistically different at 65 days of survival ($p < 0.04$). However, neither $RAD_{23–31}$ and $RAD_{24–31}$ peptide vaccination alone nor combined with anti-CTLA4 Ab treatment did delay tumor take in this experiment.

Discussion

Vaccination with a mixture of two immunogenic RAD50-peptides, $RAD_{23–31}$ and $RAD_{24–31}$, detected among six H-2^{b} binding ones, had some protective capacity in mice against tumor take following a subcutaneous inoculation of 10^6 SM7 thymoma cells. Memory for tumor rejection antigens did develop in only 3 of 5 of the tumor-rejecting mice as evidenced by the absence of tumor take after a second tumor challenge. Vaccination with a mixture of four immunogenic peptides derived from other potentially up-regulated SM7 proteins did not induce tumor protection. The present and previous experiments [8] might suggest that differences in mRNA expression profiles could be an efficient way to search for tumor rejection epitopes. However, the best interpretation of the present data is that such epitopes are weakly and inconsistently expressed by the tumor cells from which the peptides are derived. This interpretation is based on following reasoning:

Firstly, we were unable to reproduce our previous observations of prolonged survival and decreased tumor take in mice after immunization with the pool of six RAD50 peptides [8]. This inconsistency might reflect differences between the tumor cells used for challenge in our former and present study, respectively. Thus, the SM7 tumor cells used in [8] were derived from a freshly obtained solid tumor subcultured for 8–10 passages in vitro, whereas the SM7 cells of the present study was derived from a frozen stock of the first in vivo passage of SM7 cells subcultured for 3–5 passages in vitro.

Secondly, partly overlapping CTL activities were generated after peptide vaccination with the two protective closely related immunogenic RAD50 peptides (see Table 1), but these CTLs were unable to kill SM7 tumor cells in vitro. This observation might suggest either that the rejection epitopes are not identical with the RAD50 peptides used for vaccination or that RAD50 epitopes are being expressed in vivo only. Thus at best, RAD50-derived epitopes are only weakly and inconsistently expressed. A similar inconcistency between lack of killing of tumor cells in vitro and protection against tumor take was reported recently after immunization with wild-type p53 expressing vaccinia virus [15]. Protection in this latter work was shown to involve both CD4, CD8 and NK cell responses.

Thirdly, CTLA4 blockade, which suppresses inhibitory costimulatory signals in responder T cells [10], tended to delay the rejection of inoculated SM7 tumor cells in naïve mice (Fig. 5). However, CTLA4 blockage failed to improve survival in $RAD_{23–31}$ and $RAD_{24–31}$ vaccinated mice, although a protective collaboration between vaccination

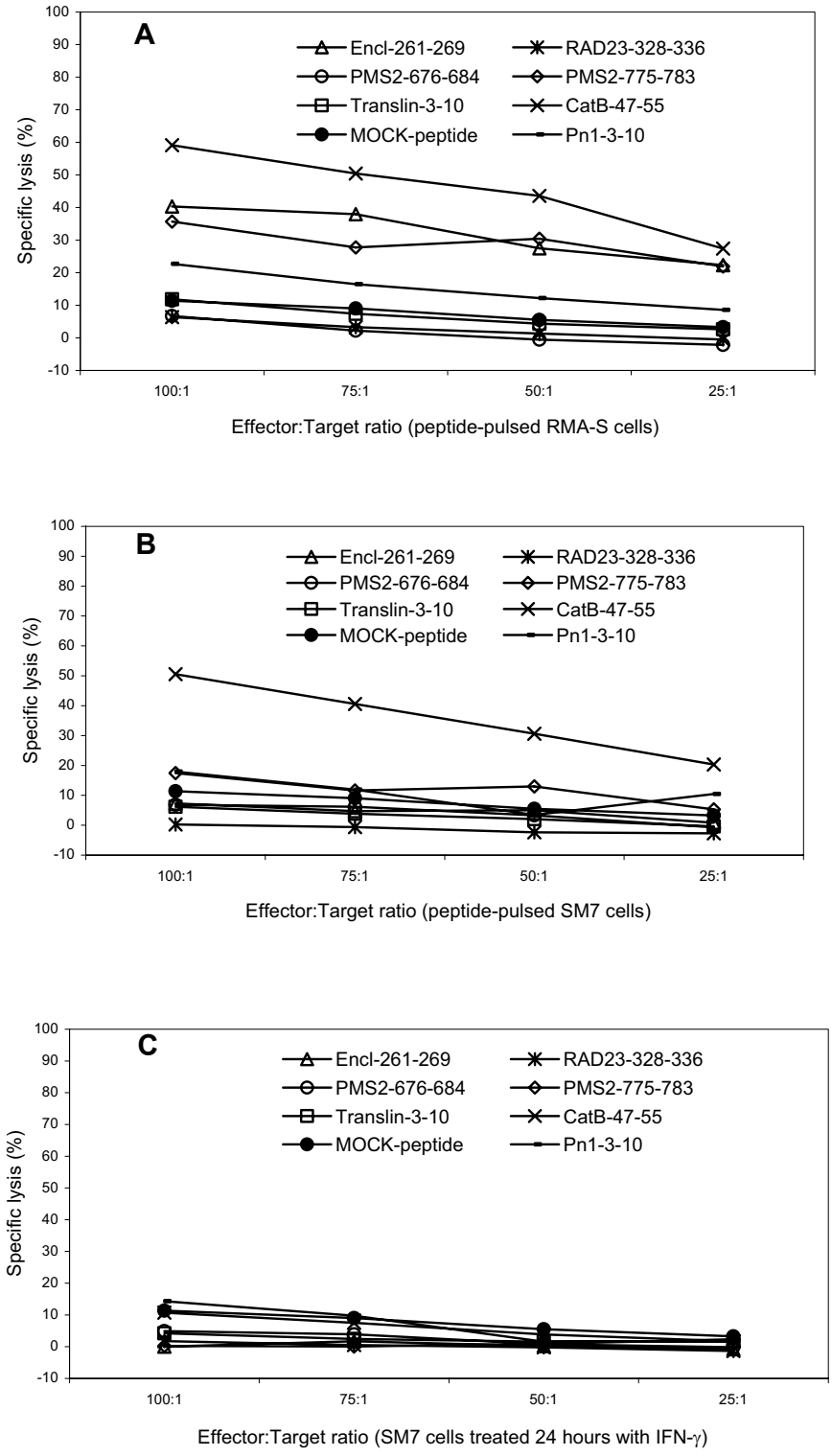

Figure 2
CTL development in vivo against SM7-derived peptides not related to RAD50. Vaccinations as described in text Fig. 1. A, Generation of CTLs against SM7 peptides. B, CTL reactivity SM7 thymoma cells pulsed with SM7 peptides. C, CTL reactivity against non-pulsed SM7 thymoma cells.

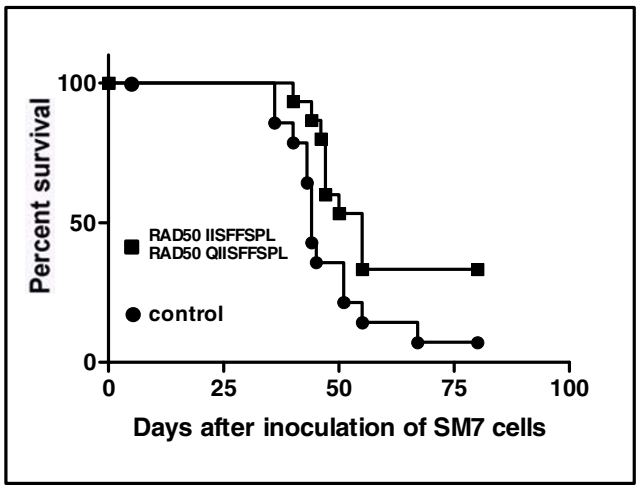

Figure 3
Survival of naïve and $RAD_{23–31}$/ $RAD_{24–31}$ vaccinated mice inoculated subcutaneously with 10^6 SM7 tumor cells. The curves represent the pooled data from two separate experiments with 15 mice per group. The curves are significantly different, $p < 0.03$. The mean survival time for controls and vaccinated mice was 44 and 55 days respectively.

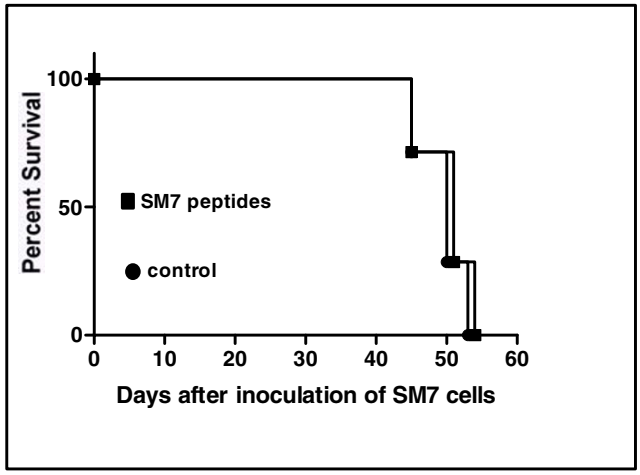

Figure 4
Survival of naïve and SM7 peptide vaccinated mice inoculated subcutaneously with 10^6 SM7 tumor cells. The curves represent data from one single experiment with 7 mice per group. The mean survival time for controls and vaccinated mice was 50 and 51 days respectively. Similar results were obtained in another separate experiment.

and CTLA4 blocking has been demonstrated previously [11,12,16]. The discrepancy in outcome between the present and the cited studies might stem from the fact that the latter studies used irradiated tumor cell vaccines, which probably are more broadly recognized by the immune system than vaccination with only a few peptides. The discrepancy also suggests that the two RAD50-derived epitopes used for vaccination, although immunogenic, do not represent important rejection epitopes for SM7 tumor cells.

Finally, the observation that only three of five vaccinated and protected mice developed memory for tumor rejection after a second tumor challenge, suggests that the $RAD_{23–31}$ and $RAD_{24–31}$ peptides are at best weak rejection epitopes which would be assumed to result in generation of weak memory.

It is unclear why vaccination with the two immunogenic peptides ($RAD_{23–31}$, $RAD_{24–31}$) alone showed protection, but lacked protection in the present study when mixed with the four non-immunogenic peptides in the RAD50 peptide mixture. Because the four nonimmunogenic RAD50 peptides exhibit higher binding affinities for H-2^b than the two immunogenic peptides [13], we specifically addressed the potential problem of peptide competition for MHC-I binding during the vaccination process. CTL generation in mice immunized with the immunogenic peptides was compared with CTL generation in mice immunized with a mixture of immunogenic and nonimmunogenic peptides was compared. We found no evidence for that CTL generation was not affected by the admixed nonimmunogenic peptides (data not included).

Why did only two of the six selected and immunogenic peptides induce protection after vaccination? The present program for prediction of peptide binding to MHC-I also includes a prediction for efficient peptide processing [13], but the power of prediction is not 100%. The possibility therefore exixts, that the immunogenic, but nonprotective peptides, of the present study are not naturally processed and such peptides would, although immunogenic, be irrelevant for tumor rejection. In addition, the thymoma cells themselves apparently express a high resistance to CTL-mediated killing since peptide-pulsed SM7 cells were killed by only two of six CTL lines capable of killing peptide-pulsed RMA-S cells.

CTL generation after immunization was achieved with less than half of the selected, H-2^b binding peptides. The CTL responses obtained were relatively weak and might reflect low TCR affinities for these self peptide epitopes due to deletion of T cells with high affinity TCRs during the process of central tolerance to ubiquitously expressed

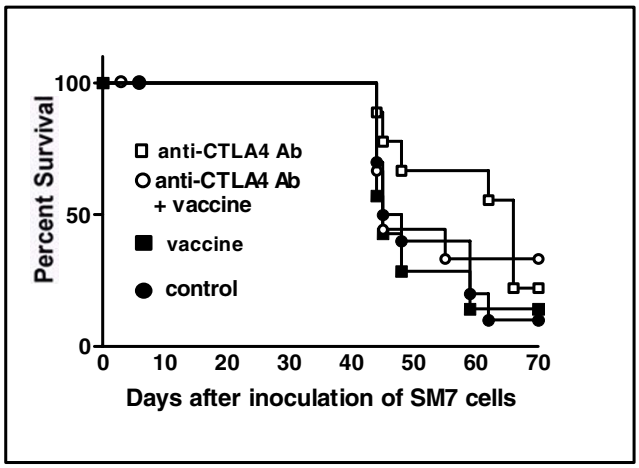

Figure 5
Survival of and tumor growth in naïve and anti-CTLA4 treated mice inoculated subcutaneously with 10^6 SM7 tumor cells. The curves represent data from one single experiment with 9–10 mice per group. The curves representing control mice and anti-CTLA4 Ab treated mice are significant different, $p < 0.04$.

self proteins [13]. Thus additional measures to increase anti-tumor responses after vaccination with these peptides should include co-administration of agents that provide CD^+ T cell-like costimulatory signals such as agonistic antibodies to CD40L and /or depletion of regulatory T cells subsets e.g. anti-$CD25^+$ $CD4^+$T cells [16–18]

The observed, relatively low tumor protection, low memory for rejection and lack of direct CTL-mediated tumor cell killing in vitro after vaccination with $RAD_{23-31/24-31}$ peptides suggest that protection is mediated by a mixture of nonspecific and specific mechanisms including macrophages, NK cells, CD4+ cells and CTLs as also observed in other studies [14]. We conclude that the present vaccination procedure using subcutaneous injection of potentially upregulated thymoma-derived peptides, identified by mRNA expression profiling of $p53^{-/-}$ thymoma cells, and mixed with a tumor irrelevant helper peptide in FIA, induces only weak tumor protection with incomplete memory. Thus, the present data question the efficiency of using mRNA expression profiling to identify new tumor rejection epitopes.

Materials and methods

Mice and cell cultures

Female C57Bl/6 mice were purchased from Bomholtgaard (Ry, Denmark). All mice were kept in a controlled microbial environment at The Department of Experimental Medicine at The Panum Institute (Copenhagen, Denmark) and used at the age of 6 – 8 weeks.

All cell lines were kept as continuous cultures in RPMI-1640 medium (Gibco-BRL, Rockville, MD, USA) supplemented with 10 % fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, and penicillin and streptomycin (referred to as CM). Splenocytes were obtained by squeezing the spleen gently through a metal net. A subline of the $p53^{-/-}$ SM7 thymoma described in [8] was used. These cells were derived from a frozen stock of SM7 cells subcultured for 3–5 passages in vitro after one in vivo passage.

The transporter associated with antigen processing (TAP)-deficient RMA-S cell lack the genes encoding TAP 1 and 2 proteins. RMA-S is a subclone of RMA [19]. β_2-micoglobulin (β_2m) was a kind gift from Dr.M.H. Nissen, University of Copenhagen.

Peptides

The peptides studied in the present work, represent derivates from potentially upregulated proteins of a thymoma with a spontaneously origin in $p53^{-/-}$ mice [8]. The proteins, peptides and their measured K_D values for binding to H-2^b a were dealt with in detail previously [13] and are depicted in table 1. These peptides are predicted as being naturally processed and express binding affinities for H-2^b in the 2–9800 nM/ml range [13]. A H-2^b binding MOCK peptide, representing amino acids 324–332 (FARGNYPAL) of the Sendai virus protein, was used as a control peptide in the cytotoxicity assays (see below). A T-helper peptide, representing amino acids 128–140 (TPPAYRPPNAPIL) of the hepatitis B virus core protein [14] was used in the peptide immunization protocol (see below). All peptides were synthesized by Schafer-N (Copenhagen, Denmark). Peptides were dissolved in phosphate-buffered saline (PBS) with 0.1% ammonium hydroxide and adjusted to physiological pH by adding hydrochloric acid. For immunization and for pulsing of target cells, the peptides were further diluted in PBS.

Antibodies and Interleukin 2

The specific anti-H-2^b antibody 8F12 was derived in our laboratory [20] and used as a culture supernatant of 8F12 hybridoma cells. The hybridoma 9H10 (kindly provided by Dr Rienk Offringa) and has been described previously [21] and generation of anti-CTLA-4 antibody was performed by hybridoma cultivation in Integra CL 350 (Integra Biosciences). Control hamster IgG was purchased from Jackson ImmunoResearch Laboratories. FITC-conjugated rabbit anti-mouse IgG (RAM-FITC) was purchased from DAKO (Copenhagen, Denmark). Human recombinant IL-2 was purchased from Chiron (Emeryville, CA, USA).

FACS analysis of H-2^b expression on SM7 thymoma cells

For measuring the H-2^b expression the SM7 cells were stained by indirect immunofluorescence. The primary antibody 8F12 (pan-specific anti MHC class I antibody) was added, the cells were incubated for 30 min on ice and washed once with cold PBS. Then RAM-FITC was added, and the cells were incubated for another 30 min on ice. Subsequently the cells were washed once with cold PBS and analysed in a FACScan flow cytometer (Becton-Dickinson, Mountain View, California, USA).

Immunization of C57Bl/6 mice and propagation of CTLs in vitro

The C57Bl/6 mice were injected subcutaneously (s.c.) in the right flank with 30 μg peptide and 120 μg T-helper peptide emulsified in 50 μl PBS and 50 μl Freunds incomplete adjuvans (FIA) (Statens Serum Institut, Copenhagen, Denmark). After 8 days, spleens of immunized mice were removed and splenocytes were cultured in 24-well plates with 5 × 10^6 immunized splenocytes and 5 × 10^6 peptide-pulsed, irradiated syngenic splenocytes from non-immunized mice per well in a total volume of 1 ml CM. After 1 day of culturing 20 U/ml IL-2 were added. After 5 days the cultured responder splenocytes were harvested, pooled, counted and cultured in four replicates of 200 μl round-bottom 96-well microculture plates at titrated effector to target (E:T) ratios of 100:1, 50:1, 25:1 and 12.5:1 (see below).

Cytotoxicity assay

The cytotoxicity of the generated CTLs was tested in a 4 hrs chromium release assay. As targets, RMA-S cells were pulsed either with the immunizing peptide or with the MOCK peptide. The RMA-S cells were pulsed for 1 h with 30 μg peptide/ml and 10 μg β_2m/ml. In addition to these RMA-S cells SM7 cells were used as targetcells. For ^{51}Chromium (^{51}Cr) labelling the target cells were incubated for 1 h at 37°C in 100 μCi sodium ^{51}Chromate ($Na_2\ ^{51}CrO_4$, Amersham, Bucks, UK) containing 30% FCS. The ^{51}Cr-labeled target cells were then washed three times and 2 × 10^3 cells in 100 μl were added to the effector cells giving a total volume of 200 μl, and incubated for 4 h at 37°C in 5% CO_2. ^{51}Cr-release was measured in 30 μl supernatant using a gamma-counter (Wallac 1470 Wizard™, Turku, Finland). Spontaneous and total release were measured by adding CM and 1% acetic acid, respectively. The percentage of specific lysis (SL) was calculated from the following formula:

$$\%\ \text{specific lysis} = 100 \times \frac{\text{esperimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}$$

Vaccination, anti-CTLA4 Ab treatment and tumor challenge

Groups of 7–10 C57BL/6J mice were vaccinated three times s.c. at weekly intervals with a mixture of six RAD50, a mixture of RAD_{23-31} and RAD_{24-31} peptides and a mixture of four immunogenic peptides derived from other potentially upregulated SM7 proteins (see Table 1). The vaccines contained 30 μg of each experimental peptide and 120 μg T-helper peptide in 50% FIA and 50% PBS per mouse. Control mice only received FIA containing T-helper peptide. One week after the last vaccination, all mice were challenged s.c. with 10^6 SM7 cells. The mice were palpated once to twice a week and killed, when the tumors reached 1 × 1 cm in size, or when they were moribund. Vaccinated and naive mice were treated with intraperitoneal injections of 100 μg anti-CTLA4 antibody at the same day as tumor inoculation and 50 μg Ab day 3 and day 6 post tumor inoculation.

Statistictical Analysis

To analyze survival data a logrank test (Prism) was used.

Abbreviations

CTL: Cytotoxic T cells, **MHC-I:** MHC class I antigens, **TAA:** tumor associated antigens, **CTLA4**: cytotoxic T lymphocyte antigen

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